

Activin A Is a Prominent Autocrine Regulator of Hepatocyte Growth Arrest

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Activin A, a multifunctional cytokine, plays an important role in hepatocyte growth suppression and is involved in liver size control. The present study was aimed to determine the cell location of activin A in the normal rat liver microenvironment and the contribution of activin A signaling to the hepatocyte phenotype to obtain insight into molecular mechanisms. Immunohistochemical and *in situ* hybridization analyses identified hepatocytes as the major activin A-positive cell population in normal liver and identified mast cells as an additional activin A source. To investigate paracrine and autocrine activin A-stimulated effects, hepatocytes were cocultured with engineered activin A-secreting cell lines (RF1, TL8) or transduced with an adeno-associated virus vector encoding activin β A, which led to strikingly altered expression of cell cycle-related genes (Ki-67, E2F transcription factor 1 [*E2F1*], minichromosome maintenance complex component 2 [*Mcm2*], forkhead box M1 [*FoxM1*]) and senescence-related genes (cyclin-dependent kinase inhibitor 2B [*p15^{INK4b}/CDKN2B*], differentiated embryo-chondrocyte expressed gene 1 [*DEC1*]) and reduced proliferation and induction of senescence. Microarray analyses identified 453 differentially expressed genes, many of which were not yet recognized as activin A downstream targets (e.g., ADAM metalloproteinase domain 12 [*Adam12*], semaphorin 7A [*Sema7a*], LIM and cysteine-rich domains-1 [*Lmcd1*], DAB2, clathrin adaptor protein [*Dab2*]). Among the main activin A-mediated molecular/cellular functions are cellular growth/proliferation and movement, molecular transport, and metabolic processes containing highly down-regulated genes, such as cytochrome P450, subfamily 2, polypeptide 11 (*Cyp2C11*), sulfotransferase family 1A, member 1 (*Sult1a1*), glycine-N-acyltransferase (*Glyat*), and bile acid-CoA:amino acid N-acyltransferase (*Baat*). Moreover, Ingenuity Pathway Analyses identified particular gene networks regulated by hepatocyte nuclear factor (HNF)-4 α and peroxisome proliferator-activated receptor gamma (PPAR γ) as key targets of activin A signaling. **Conclusion:** Our *in vitro* models demonstrated that activin A-stimulated growth inhibition and cellular senescence is mediated through *p15^{INK4b}/CDKN2B* and is associated with up- and down-regulation of numerous target genes involved in multiple biological processes performed by hepatocytes, suggesting that activin A fulfills a critical role in normal liver function. (*Hepatology Communications* 2017;1:852-870)

Introduction

Activin A acts as a negative regulator of hepatocyte growth and plays an important role in liver regeneration.⁽¹⁻⁵⁾ Notably, overexpression of its antagonist follistatin blocks activin A, and

either infusion of follistatin or transduction of hepatocytes with a follistatin-expressing adenovirus stimulates DNA synthesis and hepatomegaly. Activin A is therefore a homeostatic regulator that limits liver size.^(6,7)

As a member of the transforming growth factor (TGF)- β superfamily, activin A is involved in a wide variety of cell-specific processes, including growth

Abbreviations: AAV, adeno-associated virus; ActR-II, activin type II receptor; Adam 12, ADAM metalloproteinase domain 12; ALK-4, activin receptor-like kinase 4; Baat, bile acid-CoA:amino acid N-acyltransferase; CK, cytokeratin; CTGF, connective tissue growth factor; Cyp2C11, cytochrome P450, subfamily 2, polypeptide 11; Dab2, DAB2, clathrin adaptor protein; DEC1, differentiated embryo-chondrocyte expressed gene 1; DMEM, Dulbecco's modified Eagle medium; DPPIV, dipeptidyl-peptidase IV; E2F1, E2F transcription factor 1; EFT α , elongation factor-1 alpha; EGFP, enhanced green fluorescent protein; F344, Fisher344; FBS, fetal bovine serum; FoxM1, forkhead box M1; Glyat, glycine-N-acyltransferase; HGM, hormonally defined growth medium; HNF, hepatocyte nuclear factor; IHC, immunohistochemistry; IPA, Ingenuity Pathway Analyses; LV, lentivirus; mRNA, messenger RNA; NAC, N-acetyl-L-cysteine; OV-6, oval cell marker antibody; *p15^{INK4b}/CDKN2B*, cyclin-dependent kinase inhibitor 2B; Pmepa1, prostate transmembrane protein, androgen induced 1; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; rh, recombinant human; ROS, reactive oxygen species; Rrad, Ras-related associated glycolysis inhibitor and calcium channel regulator; Sema7a, semaphorin 7A; Sltc2a1, solute carrier organic anion transporter family, member 2a1; Smad, SMAD family member 2; Sult1a1, sulfotransferase family 1A, member 1; TGF, transforming growth factor; trActR, truncated activin receptor; TTR, transthyretin.

Received May 5, 2017; accepted August 31, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1106/full.

Supported by National Institutes of Health grant R01 DK090325 (to M.O.).

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arrest, cell differentiation, proliferation, apoptosis, metabolism, and immune response.⁽⁸⁾ Activin A is a dimeric glycoprotein composed of two βA subunits and binds to activin type II receptors (ActR-IIA, ActR-IIB), which recruit activin receptor-like kinase 4 (ALK-4) to phosphorylate SMAD family member 2 (Smad2) and 3. After binding to this complex, Smad4 translocates to the cell nucleus and regulates transcription of downstream target genes.⁽⁹⁾ Besides Smad-dependent signaling, noncanonical pathways, e.g., mitogen-activated protein kinase signaling, have also been associated with activin A.⁽⁹⁾

The source of hepatic activin A has been uncertain. The low expression level of activin A in normal livers^(1,3,4,10) and limited antibody specificity made it difficult to detect activin A-positive cells and determine their phenotype. Thus, despite several immunohistochemical studies,^(3,10,11) it has not been possible to clearly determine the cell localization and hence the source of hepatocyte control by activin A.

Our cell transplantation experiments showed that fetal liver stem/progenitor cells sufficiently replace hepatic tissue mass through a form of cell competition,⁽¹²⁾ a phenomenon that plays an important role in mammalian organ size control.⁽¹³⁾ Transplanted cells, therefore, expand even more efficiently in aging liver,⁽¹⁴⁾ an environment characterized by less “fit” liver cells⁽¹³⁾ and diminished regenerative capacity. In addition, we demonstrated that fetal stem/progenitor cells *in vitro* are selectively resistant to the growth-inhibitory effects of activin A, presumably a result of low activin receptor expression compared to mature

hepatocytes.⁽¹⁴⁾ We previously found that activin A-stimulated cell cycle arrest in cultured hepatocytes is associated with induction of cyclin-dependent kinase inhibitor 2B (p15^{INK4b}/CDKN2B), and increased levels of p15^{INK4b}/CDKN2B and activin A RNA both characterize aging liver.⁽¹⁴⁾

The function of activin A signaling as a “hepatostat”⁽¹⁵⁾ and a regulator of hepatic cell transplantation led us to investigate the cell and molecular mechanisms of these processes. We therefore established rigorous immunohistochemical and *in situ* hybridization detection to identify activin A-positive cells in normal liver; this demonstrated that hepatocytes represent the major source of activin A and also strongly express its receptors. This demonstration that activin A is a prominent autocrine regulator led us to investigate the direct and autocrine effects of activin A on cultured hepatocytes. Gene expression profiling of these hepatocytes revealed numerous downstream target genes involved in multiple molecular and biological functions of hepatocytes, especially growth control and metabolic homeostasis.

Materials and Methods

ANIMALS

Male dipeptidyl-peptidase IV (DPP-IV)⁺ Fisher (F)344 rats (purchased from Charles River) and F344-Tg(enhanced green fluorescent protein [EGFP]) F455/Rat Resource and Research Center (Rrrc) rats and DPP-IV F344 rats of different ages (originally obtained from the Rat Resource and Research Center,

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep4.1106

Potential conflict of interest: Nothing to report.

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University of Missouri-Columbia) were used for the studies. All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh in accordance with National Institutes of Health guidelines.

IMMUNOHISTOCHEMICAL DETECTION OF ACTIVIN A

Formalin-fixed/paraffin-embedded liver sections were deparaffinized in xylene, followed by hydration steps in graded ethanol solutions (100%, 95%, 80%). After blocking in 10% goat serum and 1% bovine serum albumin, sections were stained with rabbit anti-activin A (Proteintech) as the primary antibody. The secondary antibody was poly-horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Thermo Scientific). Antibody staining was visualized using a 3-amino-9-ethylcarbazole chromogen solution (ScyTek) and counterstained with hematoxylin. Further methods about immunohistochemistry (IHC) are described in the [Supporting Material](#).

IN SITU HYBRIDIZATION FOR ACTIVIN (INHIBIN) β A SUBUNIT

Nonradioactive *in situ* hybridization was performed as described⁽¹⁶⁾ with minor modifications. Frozen liver sections were incubated with two independent, digoxigenin-labeled, activin β A antisense riboprobes, and labeled hybrids were detected with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche). Antibody staining was detected with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt substrate (Roche) and counterstained with Nuclear Fast Red (Vector). Primers used for amplification of the activin β A sequence are shown in [Supporting Table S1](#).

LENTIVIRAL VECTORS

Lentivirus (LV) vectors were designed for delivery of the activin β A gene under the elongation factor-1 alpha (EF1 α) promoter, EGFP under the transthyretin (TTR) promoter, or carrying the truncated rat activin receptor (trActR)-IIB gene. Detailed information concerning vector production and titration can be found in the [Supporting Material](#).

CELL LINES AND LENTIVIRAL GENE TRANSFER

Two rat cell lines (kindly provided by Dr. M. Dabeva, Albert Einstein College of Medicine, Bronx, NY) were used for virus transduction and coculture studies with primary rat hepatocytes. One cell line (so-called RF1) was originally established from purified Thy-1⁺ cells derived from a hepatic nonparenchymal cell fraction. The other cell line (TL8) was originally derived from a liver with cholangiocarcinoma. Aliquots of both cell lines were transduced with the activin β A-encoding LV vector at a multiplicity of infection of 10 (RF1(+);TL8(+)). Cells without vector transduction (RF1(-);TL8(-)) were used as control cells in coculture experiments. Human Huh-7 cells were transduced with the EGFP- or trActR-IIB-encoding LV vector at a multiplicity of infection of 5. Cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

ISOLATION AND PURIFICATION OF HEPATOCYTES

Rat livers were perfused with 5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid solution, followed by 5,000 U/100 mL collagenase solution (Sigma), then excised, minced, and suspended in Hank's balanced salt solution. Cell suspensions were filtered and centrifuged for 2 minutes at 50g at 4°C. Cell pellets were washed 3 times with DMEM/10% FBS and resuspended. Aliquots of cell suspension were mixed with equal volumes of Percoll (GE Healthcare Bio-Sciences) solution (containing Percoll/10X Hank's balanced salt solution, 9:1) and centrifuged for 10 minutes at 50g at 4°C. Cell viability of purified hepatocytes was at least 90%. These cell isolates consisted of highly purified hepatocytes (97.1% \pm 0.5% hepatocyte nuclear factor [HNF]-4 α ⁺ cells; analyses not shown) without detectable SRY-related high-mobility group box (Sox)-9, cytokeratin (CK)-19, connexin (Cx)43, epithelial cell adhesion molecule (EpCAM), or CK-7, as described.⁽¹⁷⁾

COCULTURE EXPERIMENTS

At day 0, cell lines (5×10^2 to 3×10^5 cells) were plated on 24-mm transwell cell-culture inserts (pore size 0.4 μ m; Corning), placed in six-well plates, incubated in 3 mL DMEM/10% FBS for 2 days, washed

in phosphate-buffered saline thereafter (day 2), and cocultured with hepatocytes for 48 hours (see below).

At day 1, freshly isolated 1×10^5 hepatocytes were plated on collagen-coated six-well plates and incubated in DMEM/10% FBS. After about 6 hours, culture medium was replaced or switched to hormonally defined growth medium (HGM). On day 2, hepatocytes were washed in phosphate-buffered saline and cultured in triplicates without (controls) or with (after insertion of transwells) various cell numbers of RF1(+)/TL8(+)/RF1(-)/TL8(-) cells for 48 hours in 3 mL serum-free minimum essential medium containing 500 ng/mL insulin (Sigma) or HGM. On day 4, hepatocytes were lysed using Trizol reagent (Life Technologies), followed by total RNA isolation and subsequent quantitative reverse-transcription polymerase chain reaction (qRT-PCR) or Affymetrix gene analyses. Experiments were repeated 2 times.

RT-PCR, qRT-PCR, AND AFFYMETRIX MICROARRAY ANALYSES

Total RNA was extracted from cell isolates using Trizol and treated with DNase I (NEB), followed by a cleanup step using RNeasy Plus Mini/Micro Kit (Qiagen).

For RT-PCR analyses, RNA was reverse transcribed using Verso complementary DNA Synthesis Kit (Thermo Scientific). A complete list of primers with the number of cycles is shown in [Supporting Table S1](#). Complementary DNA was amplified by Choice-Taq DNA Polymerase (Denville Scientific) for 10 minutes at 95°C, followed by 23–30 cycles at 94°C for 30 seconds, 60°C for 20 seconds, 72°C for 60 seconds, and a final cycle at 72°C for 7 minutes.

qRT-PCR was performed in two independent experiments, each assayed in duplicate, using the StepOnePlus Real-Time PCR System (Applied Biosystems). Samples were analyzed using Power SYBR Green Master Mix (Applied Biosystems). Messenger RNA (mRNA) abundance was determined by normalization of the data to the expression levels of glyceraldehyde 3-phosphate dehydrogenase mRNA. RT-PCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method.⁽¹⁷⁾ Primers are listed in [Supporting Table S1](#).

Affymetrix GeneChip Rat 2.0 ST microarrays were used to determine mRNA expression levels in hepatocytes (1×10^5 cells/well) cocultured with TL8(+) cells (0.5×10^5 cells/insert) in triplicates for 48 hours in HGM and compared to cocultured hepatocytes with

TL8(-) cells. Ocean Ridge Biosciences performed amplification and hybridization of RNA samples, including data analyses (see the [Supporting Material](#)).

ADENO-ASSOCIATED VIRUS VECTOR PRODUCTION, TITRATION, AND GENE TRANSFER *IN VITRO*

Adeno-associated virus (AAV) particles were used for *in vitro* delivery of the activin βA gene in rat hepatocytes and Huh-7 cells. Detailed information can be found in the [Supporting Material](#).

DATA ANALYSIS

Data were analyzed using Excel (Microsoft Office 2010) software and are reported as mean \pm SEM.

Additional information can be found in the [Supporting Material](#).

Results

IDENTIFYING HEPATOCYTES AS THE MAJOR SOURCE FOR ACTIVIN A IN NORMAL LIVER

Despite the liver growth-inhibitory effects of activin A, it has been difficult to define its expression in normal liver. We screened several antibodies and found one with specificity and low background that enabled liver detection with a high antibody concentration (Fig. 1A; 1B, left). Even so, activin A could not be detected without enzymatic amplification, which precluded use of immunofluorescence. The specificity of activin A detection was verified by positive staining of oocytes (Fig. 1B, right). *In situ* hybridization labeling with two activin βA -specific antisense probes provided further confirmation of the immunodetection (Fig. 1C).

Analyses were carried out on normal liver tissue sections derived from 3- and 12-month-old rats. These studies weakly detected activin A in young liver sections (Fig. 1A, upper left). A stronger well-defined granular cytoplasmic staining pattern of activin A was observed in adult liver tissues (Fig. 1A, upper right). The vast majority of activin A-positive cells were hepatocytes (Fig. 1A, lower left). Furthermore, we also identified a nonparenchymal cell population that strongly expressed activin A (highlighted by

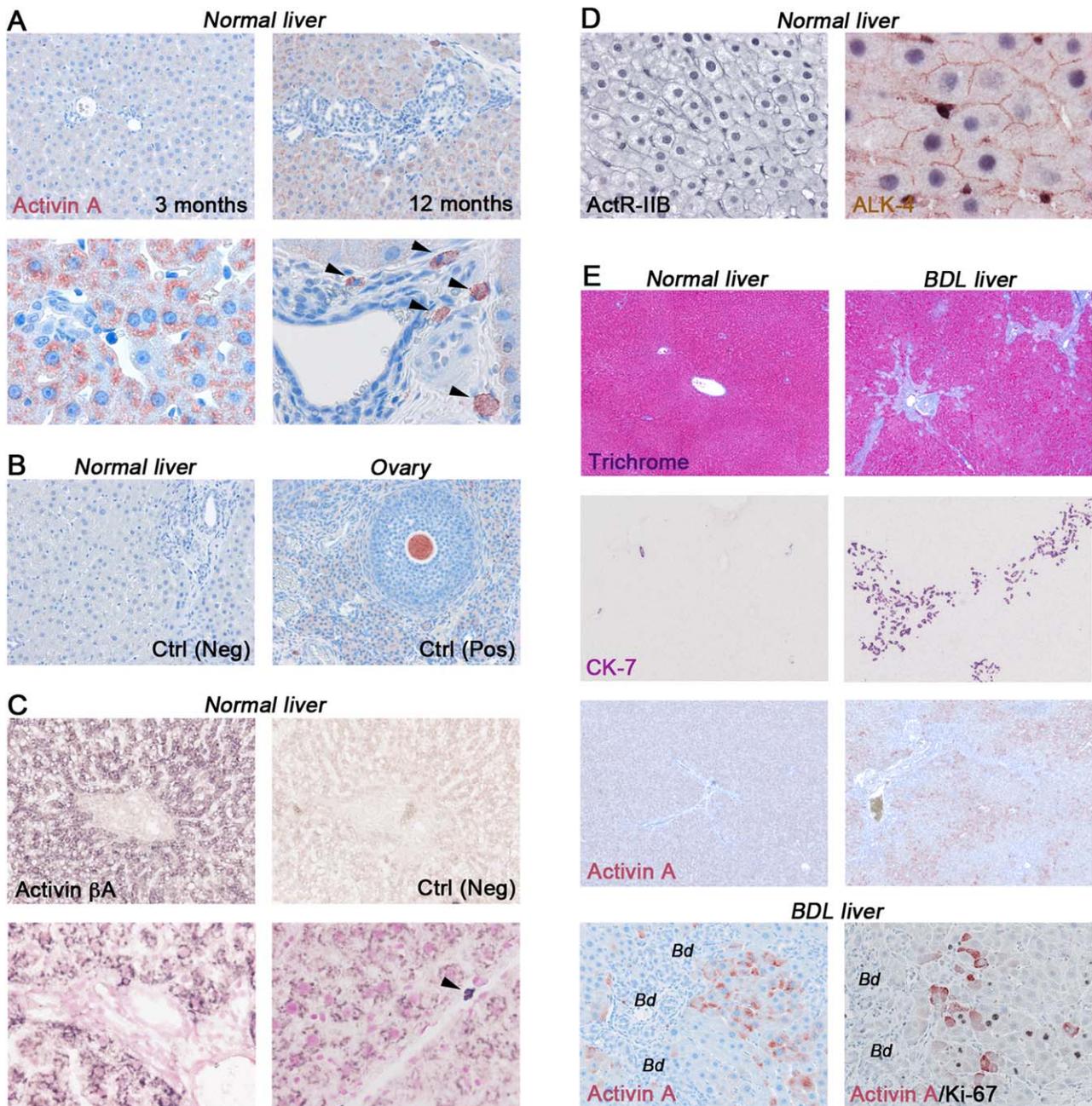


FIG. 1. Activin A and receptor expression in normal liver and changes in activin A expression in the fibrotic microenvironment. (A) Tissue sections of livers at 3 (upper left panel) and 12 months of age (upper right and lower panels) were analyzed by IHC for activin A. Expression pattern in hepatic parenchyma (lower left panel). Note the presence of activin A-positive cells in connective tissue (lower right panel, arrowheads; see also panel C, lower right panel). (B) Negative control, without primary antibody; positive control, rat ovary. (C) mRNA expression in 19- (upper panels; consecutive sections) and 12-month-old livers (lower panels) detected by *in situ* hybridization using two antisense probes specific for activin β A (probe 1, left panels; probe 2, lower right panel) or sense riboprobe (upper right panel). (D) IHC for activin receptors. (E) Biliary fibrosis was induced by BDL in 2-month-old rats. Masson's trichrome staining and IHC for CK-7 determined pathologic changes at 1 month compared to age-matched normal control rats (rows 1 and 2). Activin A expression in normal liver and at 1 month after BDL is shown (rows 3 and 4). Simultaneous IHC for activin A and Ki-67 (row 4, right panel). Note changes in activin A expression pattern and increased number of proliferating activin A-negative hepatocytes in fibrotic liver. Original magnification $\times 200$ (A,C upper panels; B; E, lower panels), $\times 640$ (A, lower panels; D, right panel), $\times 400$ (C, lower panels; D, left panel), $\times 50$ (E, rows 1-3). Abbreviations: Bd, bile duct; BDL, bile duct ligation; ctrl, control.

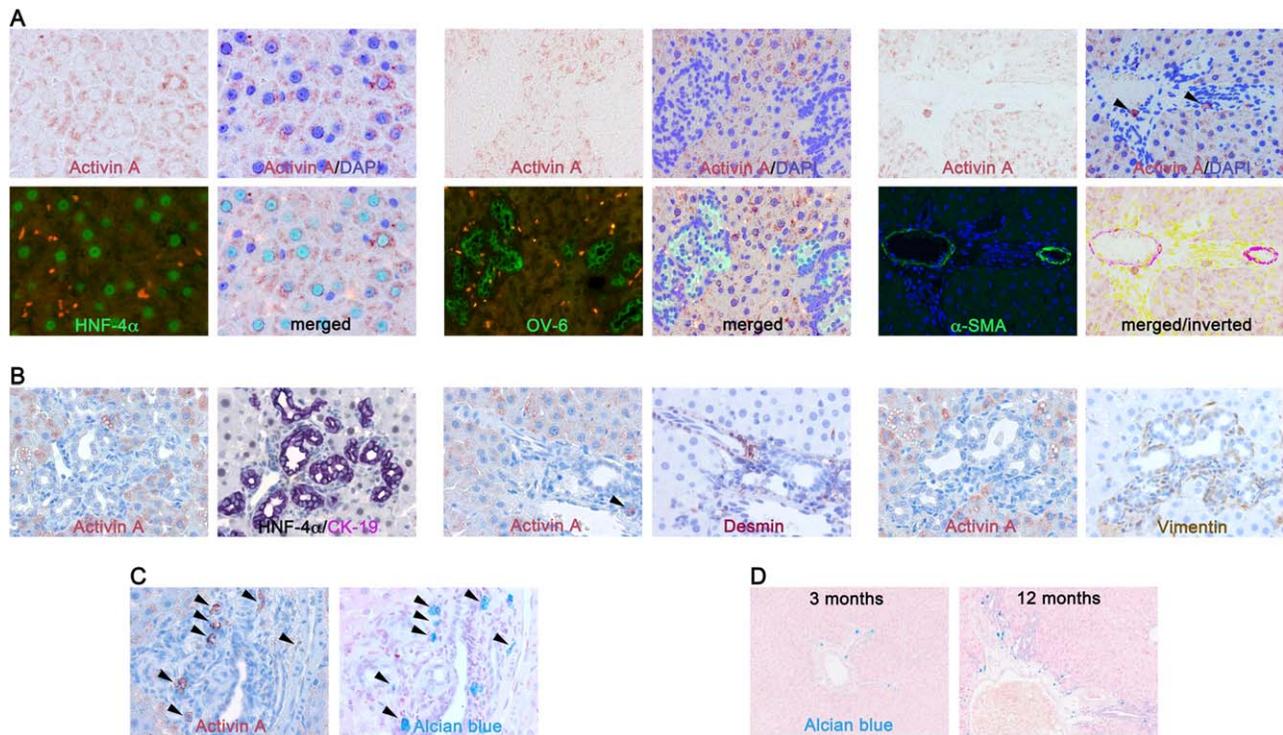


FIG. 2. Phenotypic characterization of activin A-expressing cells in normal liver. (A) Simultaneous immunohistochemical detection methods for activin A and HNF-4 α /OV-6/ α -SMA and (B) antibody stainings for activin A in comparison with cell type-specific marker expression in consecutive liver sections were used to characterize activin A-producing cells in livers at 12 months of age. (C) Activin A-positive mast cells (arrowheads; see also panels A and B). (D) Detection of mast cells in livers of different ages. Original magnification $\times 640$ (A, left panels), $\times 400$ (A, middle panels; B,C), $\times 320$ (A, right panels), $\times 100$ (D). Abbreviation: α -SMA, alpha-smooth muscle actin; DAPI, 4', 6-diamidino-2-phenylindole.

arrowheads in Figs. 1A, lower right, and 2A,B, right and middle, respectively), located in connective tissue adjacent to blood vessels and bile ducts in the central liver. Their morphology suggested that these were mast cells, which was confirmed by Alcian blue staining (Fig. 2C). A 3-fold increase in mast cells was observed in adult liver compared to young tissue samples (159 ± 34 cells/cm² versus 55 ± 8 cells/cm²; $P < 0.01$) (Fig. 2D). Mast cells are therefore an additional activin A source in normal liver. Nevertheless, because mast cells are a minor component restricted to the central liver, hepatocytes are the predominant source of activin β A mRNA in normal liver (Fig. 1C).

Activin A binds with high affinity to activin type II receptors, which are recognized by type I receptor and necessary for activation of Smad2/3 signaling.⁽⁹⁾ Our immunohistochemical analyses showed that both receptor types are strongly expressed on hepatocytes as well as on nonparenchymal cells (Fig. 1D), indicating that hepatocyte-derived activin A acts as a

prominent autocrine regulator and might have stimulating effects on mesenchymal cells in normal resting liver.

Our previous studies showed that transplanted cells require a regenerative stimulus to expand in healthy liver. In contrast, in the diseased liver environment of biliary fibrosis, transplanted hepatocytes are capable of expanding without additional treatments.⁽¹⁷⁾ Moreover, we observed a rapid increase in liver size after bile duct ligation⁽¹⁷⁾ and therefore analyzed tissue samples for activin A expression. At 1 month after bile duct ligation, the injured liver is characterized by progressive portal tract fibrosis (Fig. 1E, upper panels) and strong cholangiocyte proliferation (Fig. 1E, row 2). Importantly, we observed many regions of quiescent activin A-expressing hepatocytes adjacent to zones of actively proliferating but activin A-negative hepatocytes (Fig. 1E, rows 3 and 4). The conjunction of these two types of altered hepatocytes suggests that activin A, directly or indirectly, locally

programs both senescence and compensatory proliferation.

Additional studies evaluated the relationship of activin A-expressing cells to other liver cell types in 12-month adult liver. These analyses demonstrated that activin A-positive cells co-expressed HNF-4 α , indicating a hepatocytic phenotype (Fig. 2A,B, left), but did not express bile duct cell-specific markers, e.g., oval cell marker antibody (OV-6) or CK-19 (Fig. 2A,B, middle and left, respectively). Subsequently, we performed IHC using specific antibodies for activin A, alpha-smooth muscle actin, desmin, vimentin, which showed that stellate and mesenchymal cells do not express activin A in normal liver (Fig. 2A,B, right and middle, respectively; 2B, right).

GENERATING ACTIVIN A-PRODUCING CELL LINES

To directly study the paracrine effect of *de novo* secreted activin A *in vitro*, primary hepatocytes can be cocultured with activin A-producing cells. We therefore transduced RF1 and TL8 cells, nonhepatocyte cell lines derived from rat liver that exhibit mesenchymal or epithelial morphology (Fig. 3A), with an activin β A-encoding LV vector construct (Fig. 3B). Synthesis of activin β A mRNA was increased 13-fold and 17-fold in RF1(+) and TL8(+) cells, respectively, compared to isolated 3-month-old hepatocytes (Fig. 3C, upper panels). These changes were specific for the homodimer activin A consisting of two β A subunits because the mRNA levels of α and β B subunits, which dimerize with β A to produce inhibin A and activin AB, were unchanged in both cell lines without or with a vector construct (Fig. 3C, middle panels). No differences were detected in the expression levels of TGF- β 1 (Fig. 3C, middle panels), another TGF- β family member that shows structural similarity to activin A and can also activate Smad2/3 signaling.⁽⁹⁾ Moreover, our studies showed constitutive proliferative activity of these cells (Supporting Fig. S1), enabled by low intrinsic expression of activin receptors (ALK-4 and ActR-IIA; Fig. 3C, lower panels).⁽⁸⁾

Under normal culture conditions, 1×10^6 RF1(+) and TL8(+) cells, plated and cultured in DMEM/10% FBS for 48 hours in 75-cm² flasks, produced 91.8 ± 20.8 ng/mL and 180.0 ± 29.6 ng/mL activin A, respectively. The biological activity of secreted activin A was verified by its ability to induce hemoglobin synthesis in K562 cells (Fig. 3D). Moreover, cell line-derived supernatants were tested on Huh-7 cells,

which are responsive to activin A-mediated cell growth arrest. We then examined expression of selected cell cycle and senescence-related genes (p15^{INK4b}/*CDKN2B*, differentiated embryo-chondrocyte expressed gene 1 [*DEC1*], Ki-67, and E2F transcription factor 1 [*E2F1*]) that are up- or down-regulated in hepatocytes after treatment with recombinant human (rh) activin A.⁽¹⁴⁾ All showed robust changes (Supporting Fig. S2, upper panels); however, mRNA expression did not change in cultures with RF1(-)/TL8(-) cell-derived supernatants (Supporting Fig. S2, lower panels).

To evaluate whether the observed differences in cell cycle-related gene expression were specifically induced by *de novo* secreted activin A, activin A signaling was blocked in Huh-7 cells by direct LV-mediated gene transfer of trActR-IIB (Fig. 3B,E). qRT-PCR analyses of Huh-7 cells (trActR-IIB) cultured with conditioned medium derived from RF1(+) cells (containing 200 ng/mL activin A) for 48 hours showed no changes in p15^{INK4b}/*CDKN2B*, *DEC1*, Ki-67, and *E2F1* mRNA compared to control Huh-7 cells (Fig. 3E,F), which confirms that the activin A effects on these cells were mediated through the activin receptor. For further confirmation, Huh-7 cells were pretreated with A8301, an inhibitor of Smad2 phosphorylation, which blocked activin A-stimulated changes in cell cycle-related gene expression in a dose-dependent manner (Supporting Fig. S3). In contrast, blocking mitogen-activated protein kinase activity (mitogen-activated protein kinase kinase, p38, c-Jun N-terminal kinase) in Huh-7 cells using the kinase inhibitors PD98059 (20 μ M), SB203580 (10 μ M), and dicumarol (100 μ M) did not prevent activin A-induced changes in gene expression of Huh-7 cells (data not shown).

PARACRINE EFFECT OF *DE NOVO* SECRETED ACTIVIN A ON HEPATOCYTES *IN VITRO*

To study the effect of *de novo* produced activin A, we used activin A-producing RF1(+) or TL8(+) cells to condition the medium of hepatocyte cultures, either serum-free medium to maintain hepatocyte survival in culture or HGM to stimulate cell proliferation (Fig. 4A). Conditioning was achieved by growing different numbers of activin A-producing or control cells in transwells. A treatment of 48 hours led to a 5-fold to 8-fold increase in p15^{INK4b}/*CDKN2B* and 3-fold to 4-fold augmented *DEC1* mRNA in both media (Fig. 4B, upper panels). In contrast, a marked down-

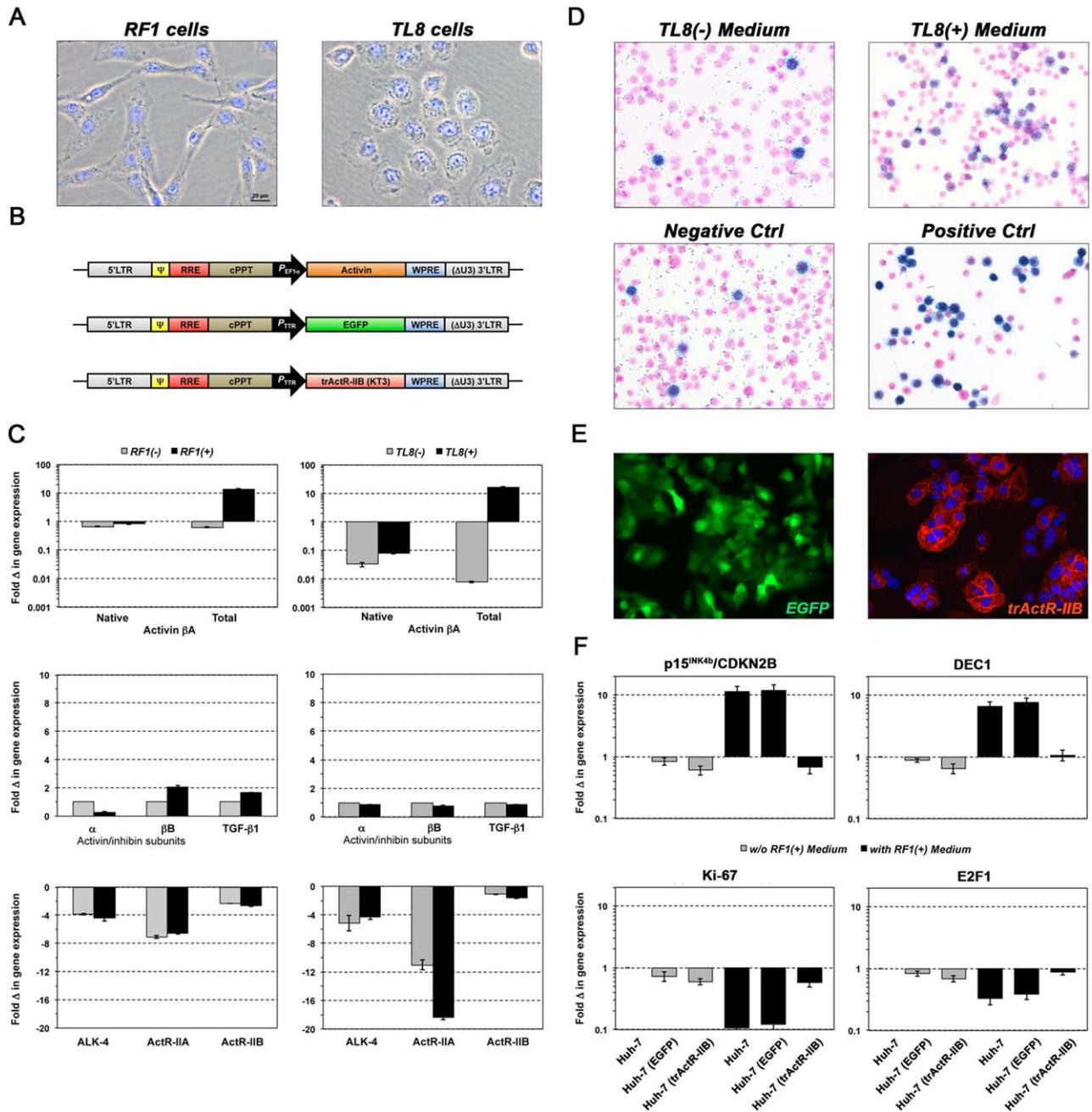


FIG. 3. Generating activin A-secreting cell lines. (A) Rat cell lines used for vector transduction. DAPI was used for staining of nuclei (blue). (B) Lentivirus vector constructs. (C) RNA extracts from cell lines (three independent cell passages each) with RF1(+), TL8(+) or without vector construct for activin βA (RF1(-), TL8(-)) were analyzed for mRNA expression of activin/inhibin subunits and activin receptors. Two replicate PCR analyses were performed. (C, upper and lower panels) Mean \pm SEM values are expressed as fold differences in gene expression compared to primary hepatocyte isolates (3-month-old rats; $n = 3$), set to a value of 1. (C, middle panels) Mean \pm SEM values are expressed as fold changes in cell lines carrying the transgene with respect to cells without the transgene. (D) Induction of erythroid differentiation in K562 cells by secreted activin A. Cells were cultured with conditioned medium derived from TL8 cells, regular medium and hemin (controls). Hemoglobin-containing cells became benzidine positive (blue). (E) After transduction with LV-EGFP or LV-trActRIIB, $> 80\%$ of Huh-7 cells were positive for the reporter gene (direct visualization of EGFP, left; IHC for KT3-tag, right). Subsequently, Huh-7 (trActR-IIB) cells were grown in culture medium in the presence of rh activin A for several passages to obtain Huh-7 cells with 100% truncated activin receptor expression. (F) Huh-7 cells were cultured in triplicates without or with conditioned medium derived from RF1(+) cells for 2 days. RNA extracts were analyzed with respect to regular Huh-7 cells cultured with nonconditioned medium, set to a value of 1. Mean \pm SEM values of two independent experiments (and two replicate PCR analyses, each) are shown. Abbreviations: Ctrl, control; cPPT, central polyurine tract; DAPI, 4',6-diamidino-2-phenylindole; LTR, long terminal repeat; RRE, Rev response element; w/o, without; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

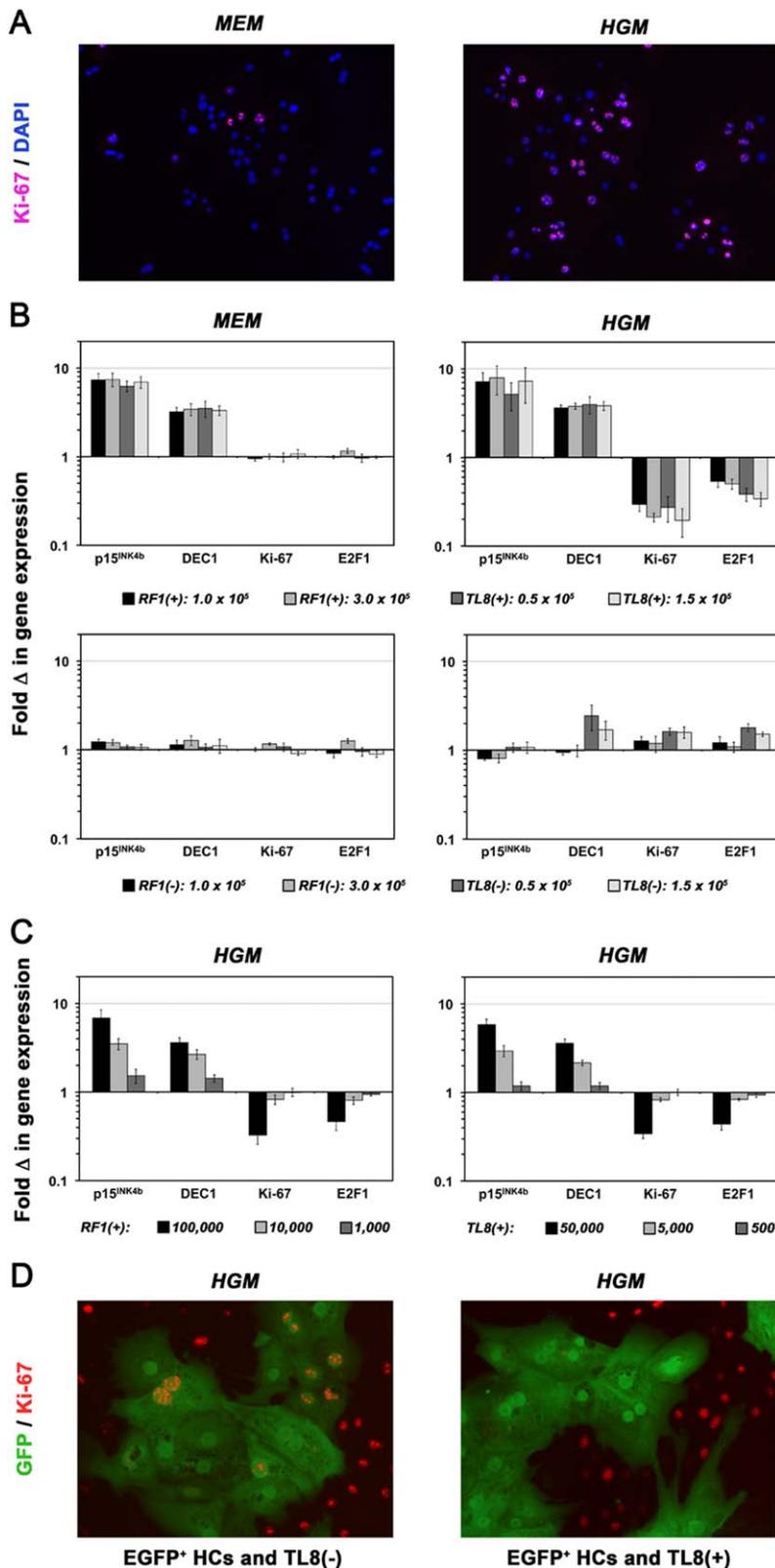


FIG. 4. Activin A-producing cell lines as a model system to study paracrine-induced growth-inhibitory effects on hepatocytes. (A) IHC for Ki-67 of hepatocytes cultured alone in different media. Note the high proliferative activity in HGM. (B,C) Induced growth inhibition by secreted activin A was determined by qRT-PCR analyses of RNA extracts from hepatocytes (1×10^5 cells/well in triplicates) cocultured with different numbers of activin A-producing cells (RF1(+), TL8(+)) or cells without vector construct (RF1(-), TL8(-)) for 48 hours in separate chambers. Mean \pm SEM values of three independent experiments (including two replicate PCR analyses each) are expressed as fold changes with respect to cultured hepatocytes without cell lines. (D) IHC for Ki-67 and GFP in mixed cell cultures of EGFP-expressing hepatocytes (1×10^5 cells/well) and TL8(+) cells or TL8(-) cells (0.2×10^5 cells/well), cocultured for 3 days. Original magnification $\times 100$ (A), $\times 200$ (D). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HCs, hepatocytes; MEM, minimum essential medium.

regulation of Ki-67 and *E2F1* (3-fold to 6-fold and 2-fold to 3-fold, respectively) was only observed in HGM (Fig. 4B, right upper panel). To rule out other changes in conditioned medium, the experiments were controlled with medium conditioned by nontransduced cells (Fig. 4B, lower panels). Because there were no differences seen in gene expression levels of hepatocytes with conditioning by 1.0×10^5 or 3.0×10^5 RF1(+) cells and 0.5×10^5 or 1.5×10^5 TL8(+) cells, activin A effects were further titrated by conditioning with smaller numbers of RF1(+) or TL8(+) cells in HGM, leading to a reduced effect on the expression of proliferation/senescence-related genes (Fig. 4C).

Direct coculture of hepatocytes and activin A-producing cells confirmed the effects of conditioned medium. IHC for Ki-67 defined a reduced proliferation of hepatocytes cocultured with TL8(+) cells compared to TL8(-) cells (Fig. 4D).

DIFFERENTIAL GENE EXPRESSION PATTERN OF CULTURED HEPATOCYTES TREATED WITH ACTIVIN A

After establishing *in vitro* conditions, RNA isolated from 1×10^5 hepatocytes cocultured with 0.5×10^5 TL8(+) or TL8(-) cells in HGM was used for microarray expression profiling to identify transcripts altered in hepatocytes by secreted activin A. We hybridized 29,489 probe sets with amplified total RNA to reveal 453 genes that showed statistically significant ($P < 0.05$) changes greater than 1.5-fold (Fig. 5A), of which 230 were up-regulated and 223 were down-regulated. The top 50 genes are listed in Table 1, and the full set is listed in Supporting Table S2. We subsequently examined mRNA expression levels of 10 selected genes by qRT-PCR, which confirmed the microarray analysis (Fig. 5B). In direct comparisons, the qRT-PCR determinations showed differences that were approximately 2-fold greater than those observed by Affymetrix analysis (Supporting Table S3).

To uncover activin A-mediated molecular mechanisms, functional analyses of differentially expressed genes were carried out using the David Bioinformatics Resource and Ingenuity Pathway Analyses (IPA). Out of 453 differentially expressed genes, 385 genes were recognized by David analysis in eight Kegg pathways, including a weak association with TGF- β signaling (seven genes; $P = 0.019$). To further elaborate the signaling pathway, we compared the hepatocyte expression to a profile of activin A-regulated genes in

embryonic stem cells,⁽¹⁸⁾ which revealed only three common genes. In contrast, comparison to profiles of TGF- β -regulated genes in renal epithelium⁽¹⁹⁾ and lung and breast cancer cell lines⁽²⁰⁾ showed 85 and 29 common genes, respectively (Supporting Table S4). Overall, 100 of the activin A-regulated genes of hepatocytes were common to at least one of the TGF- β profiles. Between the two pathways, 65 genes showed the same direction of regulation for TGF- β and activin A, while 35 showed opposite regulation. Because none of the regulations common to the two TGF- β studies showed opposite effects, the discordances suggest differences between activin A and TGF- β signaling. The cancer cell study⁽²⁰⁾ also derived a 74-gene signature for TGF- β -induced epithelial-to-mesenchymal transition, but only six activin A-regulated genes were common to this signature, a minimal relationship.

Moreover, using gene ontology analysis, 242 genes (62.9%) were categorized in several biological processes, e.g., regulation of cell proliferation, cell migration, cell cycle, and metabolic processes (Supporting Fig. S4; Supporting Table S2).

Using IPA, 427 out of 453 differentially expressed genes were mapped and functional analyses for “diseases and bio functions” were performed. The top five “molecular and cellular functions” were cellular movement, lipid metabolism, molecular transport, small molecule biochemistry, and cellular growth and proliferation (Fig. 5C). The top-ranked network of gene connectivity designed by IPA consists of 31 up- and down-regulated focus molecules (e.g., semaphorin 7A [*Sema7a*], cytochrome P450, subfamily 2, polypeptide 11 [*Cyp2C11*], glycine-N-acyltransferase [*Glyat*], bile acid-CoA: amino acid N-acyltransferase [*Baat*]), which exhibit a direct relationship to the transcription factor HNF-4 α (Fig. 5D). Other networks demonstrate the connectivity of numerous genes (e.g., laminin beta 3 [*Lamb3*], DAB2, clathrin adaptor protein [*Dab2*], coagulation factor III [*F3*], peroxisome proliferator-activated receptor gamma [*PPAR γ], epidermal growth factor receptor [*EGFR*]) that are involved in activin A-mediated regulation of lipid metabolism, molecular transport, or cellular growth and proliferation (Supporting Figs. S5 and S6).*

AUTOCRINE EFFECT OF ACTIVIN A IN HEPATOCYTES

Because the above studies focused on paracrine regulation of hepatocytes, we carried out subsequent studies to confirm autocrine regulation, which includes

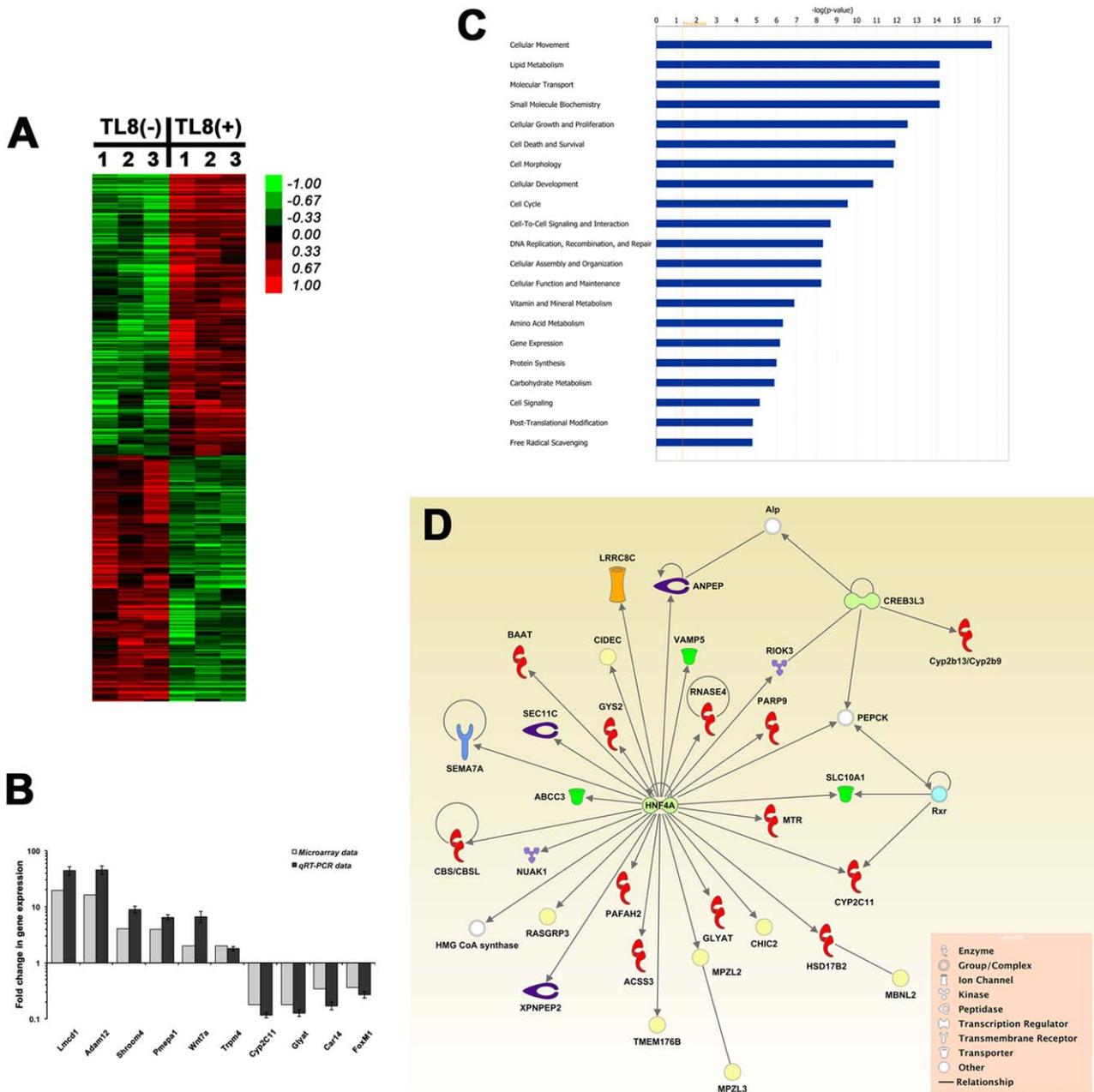


FIG. 5. Gene expression profiling in cocultured hepatocytes with activin A-secreting cells. Affymetrix microarrays were used to determine mRNA expression in hepatocytes (1×10^5 cells/well) cocultured with TL8(+) cells (0.5×10^5 cells/insert) in triplicates for 48 hours in HGM and compared to hepatocytes with TL8(-) cells. (A) Heat map containing all 453 genes that were at least 1.5-fold up- and down-regulated in cocultured hepatocytes (three independent experiments, each constellation; $P < 0.05$). (B) Validation of microarray data for selected genes. RNA extracts used for microarrays were subsequently pooled and analyzed by qRT-PCR. Changes are expressed as fold difference in mRNA expression in hepatocytes cocultured with TL8(+) versus TL8(-) cells, set to a value of 1. Mean \pm SEM values of three replicate analyses are shown and compared to microarray analysis. (C) Biological function analyses of differentially expressed genes, using IPA. (D) The top-ranked molecular network including 31 focus molecules with a score of 53 is shown. Empty symbols represent linked genes included by IPA (Additional networks in Supporting Figs. S5 and S6). Abbreviations: Abcc3, ATP binding cassette subfamily C member 3; Acss3, acyl-CoA synthetase short-chain family member 3; Alp, alkaline phenyl phosphatase; Anpep, alanyl aminopeptidase, membrane; Cbs, cystathionine beta synthase; Chic2, cysteine-rich hydrophobic domain 2; Cidec, cell death-inducing DFFA-like effector c; Creb3l3, cAMP responsive element binding protein 3-like 3; Gys2, glycogen synthase 2; Lrnc8c, leucine-rich repeat-containing protein 8C; Mbnl2, muscleblind-like splicing regulator 2; Mpz2, myelin protein zero-like 2; Mpz3, myelin protein zero-like 3; Mtr, 5-methyltetrahydrofolate-homocysteine methyltransferase; Nuak1, NUAK family kinase 1; Pafah2, platelet-activating factor acetylhydrolase 2; Parp9, poly (ADP-ribose) polymerase family, member 9; Pepck, phosphoenolpyruvate carboxykinase; Riok3, RIO kinase 3; Rxr, retinoid receptor; Sec11c, SEC11 homolog C, signal peptidase complex subunit; Slc10a1, solute carrier family 10, member 1; Tmem176b, transmembrane protein 176B; Vamp5, vesicle-associated membrane protein 5; Xpnpep2, X-prolyl aminopeptidase 2.

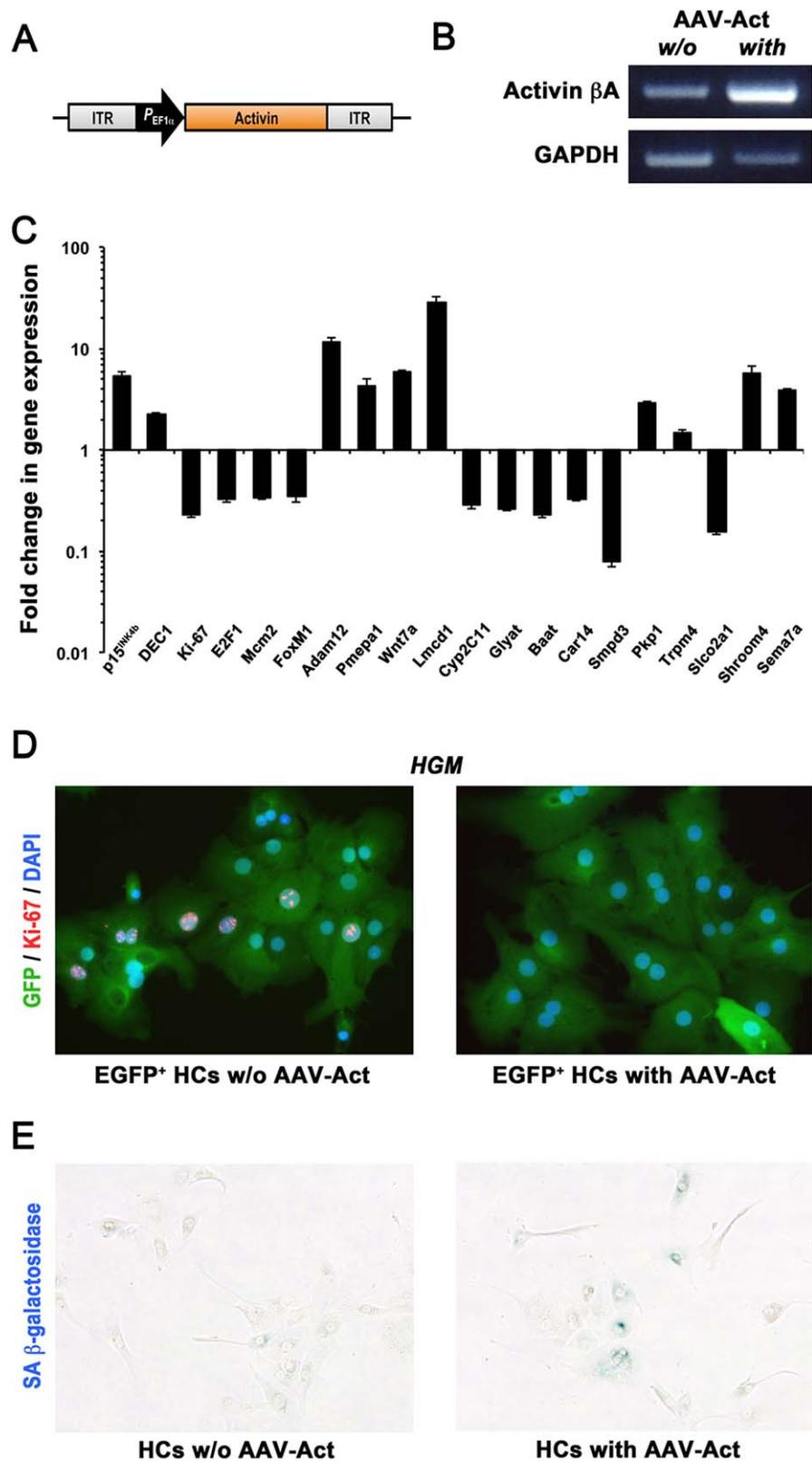


FIG. 6. Autocrine-induced effects of activin A in hepatocytes. After attachment, cultured hepatocytes were transduced with AAV-activin and analyzed 3 days thereafter. (A) Virus vector construct. (B) RT-PCR for activin βA in hepatocytes without/with vector. (C) RNA extracts from hepatocytes without/with virus were analyzed for mRNA expression of selected genes. Two replicate PCRs were performed and mean ± SEM values are shown as fold differences with respect to cultured hepatocytes without vector. (D) IHC for Ki-67/GFP in EGFP-expressing hepatocytes without or with vector construct. (E) SA β-galactosidase activity was determined in hepatocytes cultured without/with vector construct in two independent experiments. Original magnification × 200 (D), × 100 (E). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCs, hepatocytes; ITR, inverted terminal repeats; SA, senescence-associated; w/o, without.

processing and secretion of activin A in addition to its receptor-mediated signaling. For this analysis, hepatocytes were transduced with an AAV-transfer plasmid for *in vitro* delivery of the activin β A subunit (Fig. 6A,B). The effects were evaluated by qRT-PCR analysis of a panel of genes revealed by the expression profiles (Fig. 6C). All showed comparable changes and thus confirmed the microarray analysis (Table 1A,B; Supporting Table S3).

Immunohistochemical analyses demonstrated a considerable reduction of Ki-67-positive cells in hepatocytes transduced with the activin A-encoding vector compared to wild-type hepatocytes (Fig. 6D). Moreover, activin A expression led to induction of β -galactosidase expression, a marker of senescence (Fig. 6E). To substantiate the senescent phenotype, we examined transduced Huh-7 cells for β -galactosidase activity and expression of *Glb1* RNA, which encodes lysosomal β -D-galactosidase⁽²¹⁾ (Supporting Fig. S7A,B). Because accumulation of reactive oxygen species (ROS) has been shown in TGF- β -induced senescence,⁽²²⁾ we further investigated the role of ROS during activin A-induced growth arrest. Huh-7 cells were cultured with conditioned medium derived from activin A-producing RF1(+) cells (containing 100 ng/mL activin A) and treated with ROS scavenger (10 mM N-acetyl-L-cysteine [NAC]).⁽²²⁾ Activin A alone caused significant inhibition of growth and Ki-67 expression, whereas cells cotreated with NAC exhibited normal proliferation levels (Supporting Fig. S7C,D). Moreover, qRT-PCR analyses showed that NAC blocked activin A's induction of p15^{INK4b}/*CDKN2B* (Supporting Fig. S7E). ROS therefore act as a key intermediate in activin A-induced growth arrest.

These and our previous study⁽¹⁴⁾ provided strong evidence that activin A stimulates growth arrest and senescence through p15^{INK4b}/*CDKN2B* accumulation in hepatic cells. To verify that p15^{INK4b}/*CDKN2B* represents the major target gene of activin A-mediated cell cycle arrest, we transduced Huh-7 cells with an AAV-p15^{INK4b}-transfer plasmid (Supporting Fig. S8A) and demonstrated significant down-regulation of the proliferative activity in Huh-7 cells by qRT-PCR and IHC (Supporting Fig. S8B-D). Moreover, vector-induced overexpression of p15^{INK4b}/*CDKN2B* induced senescence-associated β -galactosidase activity, consistent with permanent cell cycle arrest (Supporting Fig. S8E).

Discussion

In the present study, which was focused on the role of activin A signaling in a normal hepatic

microenvironment, we made three major observations. First, our results demonstrated that hepatocytes represent the major cell source of activin A synthesis and strongly express activin type I and II receptors. Mast cells were a minor additional activin A-producing cell population in normal liver. Second, we developed *in vitro* model systems that enable study of the contribution of *de novo* synthesized activin A (secreted by neighboring epithelial/mesenchymal cells or hepatocytes themselves) to the hepatocyte phenotype. The data demonstrated that growth inhibition of hepatocytes is induced through autocrine/paracrine activin A-regulated mechanisms, reflected by strikingly changed mRNA expression of proliferation and senescence-related genes, reduced proliferation capacity, and induction of senescence mediated by ROS and p15^{INK4b}/*CDKN2B*. In addition, microarray analyses characterized numerous downstream target genes of activin A involved in growth inhibition and several other biological functions of hepatocytes, all linked through the transcription factor HNF-4 α (see Fig. 7).

After developing sensitive immunohistochemical detection and *in situ* hybridization techniques, activin A expression was weakly observed in young liver but evident in adult tissue samples, which is consistent with our previous findings using western blot analyses.⁽¹⁴⁾ Immunohistochemical analyses revealed a well-defined granulated activin A staining pattern in the cytoplasm of HNF-4 α ⁺ hepatocytes (see Figs. 1A, lower left, and 2A, left). These data imply that activin A is primarily synthesized by hepatocytes and acts as an autocrine effector molecule in normal liver, which was shown in cultured primary hepatocytes after stimulation with epidermal growth factor or hepatocyte growth factor.⁽¹⁾ There was no evidence of activin A expression in biliary epithelial/progenitor cells (CK-19⁺, OV-6⁺) or in mesenchymal cells expressing alpha-smooth muscle actin or vimentin. Furthermore, desmin⁺ cells were not identified as activin A-producing cells in the normal hepatic environment, which is consistent with studies by de Bleser et al.⁽¹¹⁾ Notably, our immunohistochemical analyses uncovered mast cells as an additional cell source for *de novo* synthesis of activin A in the liver, a phenomenon observed in these immune cells after cell activation.⁽²³⁾

Various primary hepatocyte culture models have been developed to study cellular functions, drug metabolism, and transporter interactions. Recombinant activin A has been used to study the effect of activin A on cultured hepatocytes.^(1,2,10,14) However, these models lack the influence of neighboring

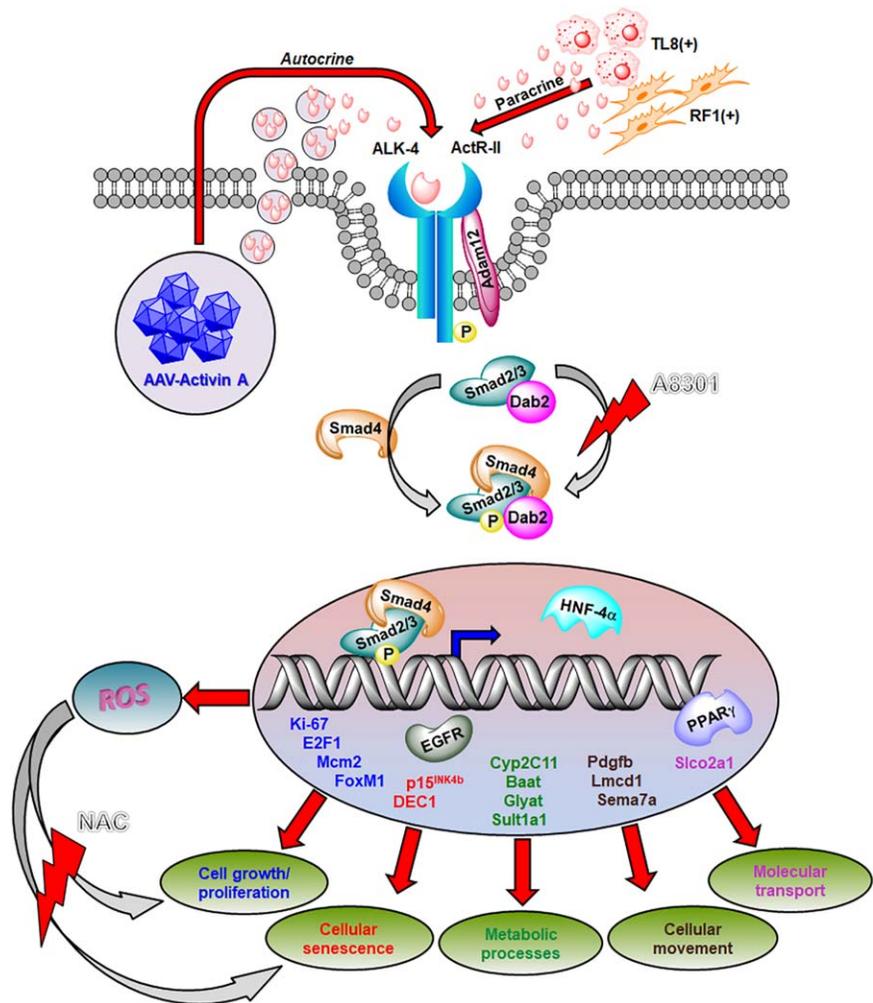


FIG. 7. Determining the general contribution of activin A signaling to the hepatocyte phenotype *in vitro*. We established two rat cell lines (RF1, TL8) carrying the gene for the activin β A subunit to secrete bioactive activin A. To evaluate paracrine or autocrine activin A-stimulated effects mediated through Smad2/3 signaling, hepatocytes were cocultured with RF1 or TL8 cells or transduced with an AAV vector carrying the activin β A subunit, which led to strikingly altered gene expression. Identified by microarray analyses, among the main molecular and cellular functions are cellular growth/proliferation, metabolism, and molecular transport. The top-ranked IPA-generated networks of gene connectivity consist of up/down-regulated focus molecules, which exhibit a direct relationship to the transcription factors HNF-4 α and PPAR γ , as well as EGFR. Abbreviations: EGFR, epidermal growth factor receptor; Pdgfb, platelet-derived growth factor β ; PPAR γ , peroxisome proliferator-activated receptor gamma.

nonparenchymal cells or their secreted factors that can stabilize or improve hepatocytic function *in vitro*.⁽²⁴⁾ A mixed monolayer of cocultured cell populations enables direct contact between cells and secreted soluble factors, although this approach is associated with technical difficulties. Because we focused on paracrine interactions rather than on direct cell–cell contact, we selected an indirect coculture approach, which permits more reliable *in vitro* results.⁽²⁵⁾ Besides human activin A-producing cell lines, rat cell lines were established from basophilic leukemia mast cells and vascular smooth muscle cells, but additional stimulation is required to release sufficient amounts of bioactive activin A into the culture media.^(23,26) Hence, we generated activin A-producing cells, which were derived from rat cell lines of mesenchymal and epithelial

origin, and studied the paracrine effect on hepatocytes cocultured in transwells. Moreover, because we identified hepatocytes as the main activin A-producing cell population in the normal hepatic microenvironment, we subsequently transduced hepatocytes with an AAV vector construct carrying the encoded activin A cytokine, which allowed us to explore the autocrine-stimulated action of activin A.

After establishing the coculture conditions by evaluating the expression levels of cell cycle-/senescence-related genes (Ki-67, *E2F1*, p15^{INK4b}/*CDKN2B*, *DEC1*) that were comparable to hepatocytes treated with rh activin A,⁽¹⁴⁾ we analyzed 29,489 gene level probes using Affymetrix microarrays. Because lesser correlations were reported for genes with a fold change in gene expression with <1.5-fold compared to those

TABLE 1A. DIFFERENTIAL GENE EXPRESSION PATTERN OF HEPATOCYTES COCULTURED WITH ACTIVIN A-PRODUCING CELLS COMPARED TO HEPATOCYTES IN ABSENCE OF ACTIVIN A SECRETION (UP-REGULATED GENES)

Gene Symbol	Gene Name	Gene ID	Fold Change	ANOVA P Value
<i>Lmcd1</i>	LIM and cysteine-rich domains-1	NM_001008562	19.90	0.003
<i>Adam12</i>	ADAM metallopeptidase domain 12	XM_008759968	16.20	<0.001
<i>Duoxa2</i>	Dual oxidase maturation factor 2	NM_001191965	9.00	0.044
<i>Serpinb1a</i>	Serine (or cysteine) peptidase inhibitor, clade B, member 1a	NM_001031642	8.99	0.008
<i>Sema7a</i>	Semaphorin 7A	NM_001108153	7.46	0.006
<i>Pkp1</i>	Plakophilin 1	NM_001107181	7.10	0.018
<i>Lamb3</i>	Laminin, beta 3	NM_001100841	6.29	0.015
<i>Fst</i>	Follistatin	NM_012561	5.98	0.004
<i>Cxcr2</i>	Chemokine (C-X-C motif) receptor 2	NM_017183	5.80	0.014
<i>Hsd17b2</i>	Hydroxysteroid (17-beta) dehydrogenase 2	NM_024391	5.54	0.009
<i>Chsy3</i>	Chondroitin sulfate synthase 3	XM_225912	5.18	0.001
<i>Dab2</i>	DAB2, clathrin adaptor protein	NM_024159	5.12	<0.001
<i>Pgf</i>	Placental growth factor	NM_053595	4.85	0.018
<i>IL33</i>	Interleukin 33	NM_001014166	4.50	0.019
<i>Mme</i>	Membrane metallo-endopeptidase	NM_012608	4.48	0.043
<i>Nppb</i>	Natriuretic peptide B	NM_031545	4.32	0.001
<i>F3</i>	Coagulation factor III (tissue factor)	NM_013057	4.23	0.020
<i>Pdgfb</i>	Platelet-derived growth factor subunit B	NM_031524	4.23	0.035
<i>Rrad</i>	Ras-related associated glycolysis inhibitor and calcium channel regulator	NM_053338	4.13	0.026
<i>Srx2</i>	Sushi-repeat-containing protein, X-linked 2	NM_001108243	4.12	<0.001
<i>Shroom4</i>	Shroom family member 4	NM_001191730	4.06	0.041
<i>Pmepa1</i>	Prostate transmembrane protein, androgen induced 1	NM_001107807	3.99	0.002
<i>Cldn4</i>	Claudin 4	NM_001012022	3.82	0.015
<i>Cd28</i>	Cd28 molecule	NM_013121	3.62	0.001
<i>Prg4</i>	Proteoglycan 4	NM_001105962	3.61	0.013

with >1.5-fold differences between groups based on microarray analyses,⁽²⁷⁾ transcripts with less than a 1.5-fold difference in gene expression were excluded from our analyses. The accuracy of microarray data was validated by qRT-PCR analyses for selected genes.

By selecting the transcripts with the 50 highest up- and down-regulated expression levels following activin A stimulation (see Table 1A,B), we discovered numerous genes that were not yet recognized as downstream targets of activin A. Only a few genes were previously known to be associated with activin A signaling. For instance, activin A regulates transcription of sex hormone synthesizing enzymes of the hydroxysteroid dehydrogenase (Hsd17) family.⁽²⁸⁾ Link et al.⁽²⁹⁾ uncovered that prostate transmembrane protein, androgen induced 1 (*Pmepa1*) is a novel and direct target gene of activin A-mediated Smad2/3 signaling in the brain. Moreover, we found that activin A regulates expression of genes that are known to be involved in TGF- β signaling. These include ADAM metallopeptidase domain 12 (*Adam12*), *Sema7a*, *Pmepa1*, and LIM and cysteine-rich domains-1 (*Lmcd1*), which regulate fibrogenesis and tumor progression^(19,20,30-32) (see also Supporting Table S4). Also, *Adam12* and

Dab2 facilitate receptor endocytosis, promote Smad phosphorylation, and enhance TGF- β signaling.^(33,34)

The majority of uncovered differentially expressed genes in hepatocytes mediated by activin A secretion (listed in Table 1A,B) are related to cell cycle and metabolism. Among those genes are novel downstream targets of activin A involved in inhibition of proliferation, including *Dab2*, Ras-related associated glycolysis inhibitor and calcium channel regulator (*Rrad*), and *Pmepa1* (all up-regulated) and RAS guanyl releasing protein 3 (*Rasgrp3*; down-regulated). Solute carrier organic anion transporter family, member 2a1 (*Sico2a1*), also known as a major transporter of prostaglandin E2,⁽³⁵⁾ which enhances hepatocyte proliferation,⁽³⁶⁾ is down-regulated after activin A treatment. Furthermore, diminished proliferation of hepatocytes is associated with decreased minichromosome maintenance complex component 2 (*Mcm2*) and forkhead box M1 (*FoxM1*) mRNA expression,^(37,38) confirming our previous report.⁽¹⁴⁾ Besides reduced proliferative activity, the present study demonstrated that this cytokine is capable in inducing Smad-dependent overexpression of p15^{INK4b}/*CDKN2B* and DEC1 and increased β -galactosidase activity in hepatic cells,

TABLE 1B. DIFFERENTIAL GENE EXPRESSION PATTERN OF HEPATOCYTES COCULTURED WITH ACTIVIN A-PRODUCING CELLS COMPARED TO HEPATOCYTES IN ABSENCE OF ACTIVIN A SECRETION (DOWN-REGULATED GENES)

Gene Symbol	Gene Name	Gene ID	Fold Change	ANOVA P Value
<i>Cyp2C11</i>	Cytochrome P450, subfamily 2, polypeptide 11	NM_019184	-5.62	0.028
<i>Glyat</i>	Glycine-N-acyltransferase	NM_001009648	-5.51	0.005
<i>Baat</i>	Bile acid-CoA: amino acid N-acyltransferase	NM_017300	-4.60	0.001
<i>Slco2a1</i>	Solute carrier organic anion transporter family, member 2a1	NM_022667	-4.39	0.006
<i>Smpd3</i>	Sphingomyelin phosphodiesterase 3	NM_053605	-4.36	0.003
<i>Mmp20</i>	Matrix metalloproteinase 20	NM_001106800	-4.20	0.033
<i>Sult1a1</i>	Sulfotransferase family 1A, member 1	NM_031834	-4.11	0.008
<i>Mettl7b</i>	Methyltransferase like 7B	NM_001024276	-4.07	0.003
<i>Inhbe</i>	Inhibin beta E	NM_031815	-4.01	0.011
<i>Rasgrp3</i>	RAS guanyl releasing protein 3	NM_001108009	-3.87	<0.001
<i>Cxcl11</i>	Chemokine (C-X-C motif) ligand 11	NM_182952	-3.81	0.020
<i>Spata2L</i>	Spermatogenesis associated 2-like	NM_001109133	-3.49	0.001
<i>Xpnpep2</i>	X-prolyl aminopeptidase	NM_057155	-3.47	<0.001
<i>Sult1b1</i>	Sulfotransferase family 1B, member 1	NM_022513	-3.45	0.015
<i>Clic5</i>	Chloride intracellular channel 5	NM_053603	-3.26	0.004
<i>Prss32</i>	Protease, serine, 32	NM_001106983	-3.10	0.001
<i>Mcm2</i>	Minichromosome maintenance complex component 2	NM_001107873	-3.01	0.041
<i>Hfe2</i>	Hemochromatosis type 2 (juvenile)	NM_001012080	-2.96	0.011
<i>Car14</i>	Carbonic anhydrase 14	NM_001109655	-2.94	0.034
<i>Lhfp</i>	Lipoma HMGIC fusion partner	NM_001109183	-2.93	0.039
<i>Glyat2</i>	Glycine-N-acyltransferase-like 2	NM_134330	-2.93	0.023
<i>FoxM1</i>	Forkhead box M1	NM_031633	-2.77	0.040
<i>Gstm2</i>	Glutathione S-transferase mu 2	NM_177426	-2.73	0.014
<i>Cbln3</i>	Cerebellin 3 precursor	NM_001109330	-2.69	0.007
<i>H2afx</i>	H2A histone family, member X	NM_001109291	-2.63	0.049

Hepatocytes (1×10^5 cells/well in triplicates) were cocultured with activin A-producing cells (TL8(+); 0.5×10^5 cells) or cells without vector construct (TL8(-); 0.5×10^5 cells) for 48 hours in separate chambers. Each constellation consists of three independent experiments of cocultured hepatocytes. Global gene expression profiling was performed using Affymetrix GeneChip Rat 2.0 ST microarrays. Changes are expressed as fold differences in mRNA expression in hepatocytes cocultured with activin A-secreting cells versus hepatocytes without activin A treatment. Two thousand genes were differentially expressed in hepatocytes after activin A treatment ($P < 0.05$). After exclusion of transcripts with less than 1.5-fold changes, 230 up-regulated and 223 down-regulated genes were identified. The 25 most up- and down-regulated genes are listed. All 453 genes are listed in Supporting Table S2, including the gene ontology biological processes for each gene.

indicating that cellular senescence is triggered by activin A signaling similar to TGF- β -induced p15^{INK4b}/CDKN2B-dependent senescence in hepatocellular carcinoma cell lines.⁽²²⁾ In addition, numerous highly down-regulated genes encoding proteins are involved in metabolic processes (*Cyp2C11*, *Glyat*, *Baat*, sphingomyelin phosphodiesterase 3 [*Smpd3*], sulfotransferase family 1A, member 1 [*Sult1a1*], carbonic anhydrase 14 [*Car14*]). For instance, glycogenolysis is stimulated by activin A and glucagon, which is involved in suppression of Cyp2C11 similar to that described by TGF- β 1.⁽³⁹⁾ Activin A also down-regulates *Baat*, a peroxisomal enzyme required for conjugation of bile salts,⁽⁴⁰⁾ which are signaling molecules that induce FoxM1b.⁽⁴¹⁾ Sulfotransferases (e.g., *Sult1a1*, *Sult1b1*) that catalyze the sulfonation in the metabolism of endogenous compounds, xenobiotics, and drugs are also down-regulated after activin A

stimulation. In addition, numerous downstream target genes of activin A (e.g., plakophilin 1 [*Pkpl1*], transient receptor potential cation channel, subfamily M, member 4 [*Trpm4*], *Slco2a1*, shroom family member 4 [*Shroom4*]) were related to other biological processes (e.g., cell death, transporter activity). Finally, using IPA functional analyses, 427 out of 453 differentially expressed genes were mapped and their gene connectivity was determined. HNF-4 α , PPAR γ , and EGFR, which are known to be involved in regulating hepatocyte proliferation and maintenance of lipid homeostasis,^(42,43) were identified as possible key target genes of activin A signaling in regulating these networks (see Fig. 5D; Supporting Figs. S5 and S6).

We previously investigated the effect of rh activin A on cultured hepatocytes using qRT-PCR arrays; however, these analyses were limited to 370 preselected genes.⁽¹⁴⁾ In the present study, we used microarrays to

screen 29,489 genes. Microarrays are less sensitive and more variable than qRT-PCR analyses.⁽⁴⁴⁾ For instance, connective tissue growth factor (CTGF) synthesis is strongly induced by activin A in cultured rat hepatocytes.⁽¹⁰⁾ In our microarray analysis, *CTGF* expression was 1.6-fold increased after activin A stimulation, but the change was not statistically significant. In contrast, qRT-PCR analyses showed a significant 4.1-fold increase of *CTGF* (see Supporting Table S3). Moreover, fold differences in gene expression determined by microarrays are generally less than those observed by qRT-PCR,⁽⁴⁴⁾ which is consistent with our observations (see Supporting Table S3). For this reason, 75% (43/57) of the genes identified in our qRT-PCR array studies⁽¹⁴⁾ are not listed in the data sets from Affymetrix microarray analyses (Table 1A,B; Supporting Table S2) because the fold changes were <1.5 or not statistically significant. Nevertheless, these showed consistent but moderate expression differences (data not shown).

The liver regulates many physiological processes in the mammalian organism that requires maintenance of a constant liver mass. However, chronic injury disrupts the “hepatostat” and leads to liver dysfunctions.⁽¹⁵⁾ Several studies reported that deregulation of activin A contributes to the pathogenesis of human liver disorders, e.g., alcoholic cirrhosis,⁽⁴⁵⁾ acute liver failure,⁽⁴⁶⁾ chronic viral hepatitis,⁽⁴⁷⁾ and hepatocellular carcinoma.⁽⁹⁾ Our new observations confirm that activin A is an important homeostatic regulator. The relationship to local injury is particularly intriguing because strong activin A-expression seems to program both senescence and compensatory proliferation in contiguous cells (see Fig. 1E). The relationship among activin A-expressing and proliferative cells in these regions is an important topic for further investigation.

Activin A-induced senescence is associated with accumulation of ROS and increased expression of the G1 cell cycle inhibitor p15^{INK4b}/*CDKN2B*, all features of aging. Xu et al.⁽⁴⁸⁾ suggested that activin A could serve as a biomarker of senescence because increasing levels of activin A lead to the reduced metabolic function associated with aging. Consistent with these observations, Ogrodnik and co-workers⁽⁴⁹⁾ demonstrated that senescence in hepatocytes leads to impaired fat metabolism and drives age-related steatosis. Besides this association with aging and age-related diseases, there is growing evidence that senescent hepatic cells and their secreted factors (senescence-associated secretory phenotype) have a considerable influence on neighboring cells and therefore play a

crucial role in non-age-related liver diseases.⁽⁵⁰⁾ In this regard, inhibition of activin A or its signaling in hepatocytes to eliminate senescent cells could be a new approach in treating dysfunction associated with age or chronic liver disease. The expression profiles characterized in this paper may include targets for these novel therapeutics.

In summary, we have developed *in vitro* model systems that mimic relevant features of the hepatic microenvironment and have performed a broad gene expression analysis, which provided the foundation for investigating autocrine and paracrine activin A-mediated molecular and biological functions in hepatocytes. Numerous new target genes were uncovered. These studies accomplished a better understanding of the role of activin A in regulating growth inhibition and senescence as well as its involvement in metabolic processes in hepatocytes and suggest that therapeutic interventions targeting modulation of activin A signaling exhibit great translational potential.

Acknowledgment: We thank Dr. George Michalopoulos (Department of Pathology, University of Pittsburgh) for his helpful comments and discussions throughout the studies and William Bowen for his technical advice on liver perfusion.

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